23.05.2025

Abstract

Hybridization reactions, which involve the specific pairing of complementary nucleic acid strands, are fundamental to various biological processes and biomedical applications. In an aqueous environment, these reactions are particularly significant due to the intrinsic solubility and stability of nucleic acids in water-based solutions.[1] The selective hybridization of nucleic acid sequences underlies a wide array of techniques, including DNA microarray technology, fluorescence *in situ* hybridization (FISH), biosensors, and therapeutic oligonucleotides.[2–5] For instance, DNA microarrays utilize the hybridization of fluorescently labeled cDNA to immobilize probes, enabling high-throughput gene expression analysis.[6] FISH enables the spatial visualization and quantitation of specific DNA and mRNA sequences within cells, facilitating studies of chromosomal abnormalities such as aneuploidy and the spatial organization of centromeres.[7] In therapeutics, antisense oligonucleotides and small interfering RNAs (siRNAs) employ hybridization to silence target genes, offering promising strategies for treating genetic disorders and viral infections.[8]

Despite their broad utility, hybridization reactions are sensitive to environmental conditions, particularly in aqueous solutions. Factors such as pH, temperature, ionic strength, and molecular crowding influence the kinetics and thermodynamics of hybridization. High ionic strength stabilizes duplex formation by shielding electrostatic repulsion between negatively charged phosphate backbones, whereas extreme pH can disrupt hydrogen bonding and base pairing.[9, 10] Moreover, macromolecular crowding modulates hybridization equilibria by introducing steric hindrance, thereby altering association, dissociation, and equilibrium constants.[11, 12] At low crowder concentrations (<15 wt.%), the equilibrium constant K of DNA-DNA complex formation increases by an order of magnitude,[13] whereas at higher concentrations, K decreases even up to three orders of magnitude.[14] These unintuitive changes are controlled by two counteracting effects: depletion forces and crowding-induced destabilization, whereas electrostatics adds another layer

of complexity to the system. Despite extensive studies, to this date, no physical model fully explains the interplay of these phenomena.

The first and second sections of this thesis investigate the influence of ions and oppositely charged molecules on nucleic acid association rates, as well as the impact of molecular crowding and ions on DNA-DNA interactions at equilibrium. The results combine experimental data with theoretical models. The first model predicts association rates of like-charged reactants in the presence of oppositely charged catalyst molecules in aqueous solutions. The second model elucidates the role of molecular crowding in modulating DNA-DNA hybridization and the interplay between electrostatics, depletion interactions, and crowding-induced destabilization.

To study DNA-DNA interactions at equilibrium, Förster Resonance Energy Transfer (FRET) and Molecular Brightness Analysis (MBA) are employed. These techniques allow the monitoring of equilibrium shifts in response to variations in pH, ionic strength, molecular crowding, and ion concentration.[14–20] When integrated with Fluorescence Correlation Spectroscopy (FCS), they provide single-molecule resolution within femtoliter volumes. FRET offers superior accuracy due to its high signal-to-noise ratio, but it requires dual fluorescent labeling and precise positioning of dyes within 1–10 nm of interacting molecules. In contrast, MBA, with a lower signal-to-noise ratio, simplifies experimental design by eliminating the need for site-specific fluorescent labeling. However, both methods are laborious and have inherent limitations in throughput.

Determining K for a simple $A + B \rightleftharpoons AB$ reaction requires extensive dilution series, typically involving 20–30 different concentration ratios.[21] Given the sample preparation and measurement requirements, such experiments are time-consuming, often taking several hours. Large-scale studies, such as those examining pH or ionic strength dependencies, further amplify the experimental burden, as the number of required samples increases by an order of magnitude. Reducing the number of data points per K determination is undesirable, as it increases measurement error from approximately 15% (with 30 samples) to ~60% (with 10 samples). Additionally, the standard protocol typically relies on disposable well plates, which accommodate 200 µL of solution per well, leading to substantial material consumption and high costs.

Even after sample preparation, manual plate exchange is required, depending on the number of wells in the plate, further impeding efficiency.

To address these limitations, the third section of this thesis presents a microfluidic-based platform that automates both sample handling and measurement. This system streamlines experimental workflows, significantly reducing manual labor by requiring only an initial setup and microscope calibration. Once initiated, the platform operates with minimal user intervention, decreasing the total time required for K determination from approximately 180 minutes to 35 minutes. Moreover, it enhances the accuracy of MBA while reducing reagent consumption by three orders of magnitude, from $\sim$200 µL per sample in conventional protocols to $\sim$100 nL per droplet in the microfluidic system. These improvements enhance both the efficiency and cost-effectiveness of DNA hybridization studies, facilitating large-scale investigations of nucleic acid interactions under various conditions.